

Dry Column Method for the Quantitative Extraction and Simultaneous Class Separation of Lipids from Muscle Tissue¹

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ABSTRACT

A method for lipid isolation is presented that is alternative to the traditional chloroform/methanol extraction methods. This new method allows lipid isolation by solvent elution of a dry column composed of a tissue sample, anhydrous sodium sulfate, and Celite 545 diatomaceous earth ground together. To isolate total lipids, the dry column is eluted with a mixture of dichloromethane/methanol (90:10, v/v). Alternatively, the lipids may be isolated and simultaneously separated into neutral and polar fractions by a sequential elution procedure; neutral lipids free of polar lipids are eluted first with dichloromethane, followed by elution of polar lipids with the dichloromethane/methanol (90:10) mixture. The two dry column methods—*isocratic* or *sequential elution*—were compared with the traditional chloroform/methanol methods by gravimetric, thin layer chromatographic and phosphorus analyses.

INTRODUCTION

Although many methods have been proposed for the extraction of lipids from biological tissue, only two, originally proposed over two decades ago, have met with great acceptance because their use presumably allows lipid to be isolated quantitatively, unaltered and free of nonlipid contamination. These two methods—the Folch et al. (1) and the Bligh and Dyer (2)—require the use of mixtures of chloroform and methanol as the extraction solvent. Despite widespread use of these methods, work in this and other laboratories during the past several years (3-5) has shown limitations in both and in their more recent modifications (6): (a) chloroform is a suspected carcinogen; (b) excessive amounts of solvent are required; (c) the procedures are tedious and time-consuming, especially for multiple extractions; (d) use of expensive tissue homogenizing equipment is required; (e) emulsion problems often are encountered; (f) separation of the lipid into its subclasses requires subsequent chromatographic procedures.

We recently developed a dry column method for the determination of the total fat content of meat and meat products (3). The method was proposed as a replacement for the laborious Soxhlet ether extraction techniques currently used by food analysts to determine fat content. We now have found that, with some modifications, this same method can be used for the isolation of intact lipids from muscle and

adipose tissue. In this report, we show that the same values for lipid content of muscle and adipose tissue may be obtained by the dry column method as those obtained by the traditional chloroform-methanol methods, but more rapidly and without many of the limitations encountered with the latter techniques. Moreover, we demonstrate that by a sequential application of solvents to the dry column, the lipid can be extracted from the column bed and separated simultaneously into major neutral and polar fractions.

EXPERIMENTAL

Reagents, solvents and apparatus were the same as discussed previously (3). Smaller columns (16 mm id × 25 cm with 8 mm id × 5 cm drip tip) were used for extraction of 1 g tissue samples. For these smaller samples, the ratio of tissue to Celite 545 to sodium sulfate was maintained as given previously for 5-g tissue samples.

Although the procedure will accommodate minced tissue, random sampling, for the purposes of this comparative study, of 5 g of tissue from much larger tissue sources demanded initial comminution of the entire tissue. This was accomplished with ease by use of a Cuisinart Model CFP5A home food processor (Robot-Coupe S.A., Stamford, CT 06902). A 5-g sample of comminuted tissue (2 g for adipose tissue) was weighed to the nearest 0.1 mg. When multiple extractions were to be done, complete subsets of 5-g portions were set aside at the same time. Samples of 1 g size were handled similarly. Because of difficulties in

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uniform sampling, it is preferable to handle the larger sample sizes when sufficient tissue is available.

Tissue Extraction: Isolation of Total Lipid by Isocratic Elution

Tissue (5 g) was ground in a 750-ml porcelain mortar with granular anhydrous sodium sulfate (20 g) and then with Celite 545 (15 g), and the resulting mixture was packed above a 1:9 CaHPO_4 /Celite 545 trap (10 g) in a glass chromatography column (id 35 mm), as previously described (3). In this study, the columns were charged with CH_2Cl_2 /MeOH (90:10) and 150 ml of eluate was collected in a 200-ml volumetric flask, whose contents then were brought to volume so that measured aliquots could be withdrawn for subsequent gravimetric, phosphorus and chromatographic analyses. Extracts of 1-g tissue samples were collected in 25-ml volumetric flasks.

Tissue Extraction: Isolation of Neutral and Polar Lipids by Sequential Elution

This procedure is similar to that described previously (3) except that two separate solvent systems are required—dichloromethane and the 90:10 mixture already described. The column was packed in the same manner, but it was charged first with 150 ml (for 5 g of tissue) of dichloromethane instead of the 90:10 solvent mixture. At the point when the last of the dichloromethane reached the top of the column packing, the flask containing the collected eluate (neutral lipid) was replaced by a second 200-ml volumetric flask, and the column was charged with 150 ml of the 90:10 solvent mixture. The eluate was collected until the column was stripped of solvent (60-90 min). This second fraction contained the polar lipids from the tissue sample. The contents of both volumetric flasks were brought to volume, and aliquots were removed as already described.

TABLE I
Variations in Elution Solvents to Optimize Efficiency of Method^a

A. To minimize nonlipid coelution (isocratic procedure):

	Solvent	Results
CH_2Cl_2 /MeOH	80:20 (v/v)	Excessive nonlipid coelutes with lipid.
	90:10 ^b	Complete elution of lipid. Nonlipid <0.1% of tissue weight with 1:9 trap (cf. text).
	99:5	Incomplete elution of very polar phospholipids.
CH_2Cl_2 /i-PrOH	99:1	Incomplete elution of all classes of phospholipids.
	80:20	Some coelution of nonlipid. Some retention of phospholipid.
	90:10	Some retention of phospholipid. Excessive amount of solvent required.
CH_2Cl_2 /acetone	80:20	All neutral lipids eluted by 10 ml; polar lipids elute after 100 ml, but incompletely.
CH_3CN		Phospholipids and nonlipid elute first, but incompletely; neutral lipids appear after 60 ml.

B. To optimize separation of neutral lipid from polar lipid (sequential elution procedure):

Solvents, numbered in their sequential order	Results
1. CH_2Cl_2 neat, 150 ml	$\left. \begin{array}{l} \text{Essentially complete elution of neutral lipid. No phospholipid coelution.} \\ \text{Complete elution of phospholipid.} \\ \text{Trace amount (ca. 3 mg) of neutral lipid (even after excess solvent is used in prior elution step).} \end{array} \right\} \text{b}$
2. CH_2Cl_2 /MeOH 90:10, 150 ml	
1. CH_2Cl_2 /MeOH (or i-PrOH) 99.75:0.25, 100 ml	$\left. \begin{array}{l} \text{Neutral lipid elutes with trace of phospholipid.} \\ \text{Trace of neutral and polar lipid.} \\ \text{Trace of neutral and polar lipid.} \\ \text{Elution of polar lipid; coelution of some neutral lipid.} \end{array} \right\}$
2. CH_2Cl_2 /MeOH (or i-PrOH) 99.75:0.25, 50 ml	
3. CH_2Cl_2 /MeOH (or i-PrOH) 99.75:0.25, 50 ml	
4. CH_2Cl_2 /MeOH 90:10, 75 ml	

^aAll experiments done on 5-g tissue samples.

^bIndicates preferred procedure.

Thin Layer Chromatography (TLC)

Adsorbent and development systems are given in Figures 1 and 2. All TLC visualizations were accomplished first by spraying the developed plate with ninhydrin (0.3% in absolute ethanol) with subsequent warming to reveal amino-group-containing lipids and nonlipids as pink spots. The same plate then was cooled and sprayed with Phospray (Supelco, Inc., Bellefonte, PA) to visualize phospholipids as heteropolyphosphomolybdate blue spots. (Some substances used in TLC such as Phospray and chloroform are toxic and must be used with care and proper ventilation.) Finally, the plate was heated in a fume hood to reveal all the material as charred spots.

For quantitative recovery of neutral lipid contaminants from polar fractions, lipid was applied quantitatively to 20 × 20 cm 250-μ-thick plates of Silica Gel H and 5% (NH₄)₂SO₄

(L/S Redicoats; Supelco, Bellefonte, PA) using a TLC sample streaker (Applied Science, State College, PA). Development was in chloroform/methanol/water (65:35:5), and visualization was with iodine vapor. Bands of neutral lipids were scraped, packed into chromatography columns, and eluted with dichloromethane to recover lipid from the adsorbent. Subsequent elution with dichloromethane/methanol (1:2) failed to remove any additional neutral lipid, as confirmed by examination of aliquots by TLC.

Phosphorus Analysis

Aliquots of lipid solutions were analyzed for phosphorus according to the method of Vas-kovsky et al. (7). Quantitation was accomplished by concurrent determination of seven pairs of solutions of inorganic standard KH₂PO₄ ranging in content from 0 to 3.39 μg P. Absorb-

TABLE II

Variations in Column Packing to Optimize Efficiency of Method^a

Modification	Results
A. To optimize column flow rate	
Column packed dry to height of 60-70 mm, then charged with solvents and allowed to drip without assistance ^b . Loosely compressed packing. Column packed wet (dry packing added to column filled with CH ₂ Cl ₂). Eluates drawn off under vacuum.	Column loading is rapid and facile; elution is unattended and complete within 2 hr. Channeling; incomplete elution of lipid. Polar lipids eluted with neutral lipids during sequential extraction. Occasional vapor lock; increased amounts of nonlipid coeluted; poor class resolution during sequential extraction.
B. To optimize separation of neutral from polar lipid (sequential procedure):	
Non-washed Celite 545 ^b . Acid-washed Celite 545. Celite 545 washed by solvent (CH ₂ Cl ₂ /MeOH 92:8 azeotrope; Soxhlet extraction). Fine-mesh ("Analytical grade") Celite. Celite 545 pre-equilibrated with water in water-saturated atmosphere. Celite 545 pretreated with 1% water. Celite 545 heat-treated at 600 C. NaHCO ₃ instead of Celite 545. Acid/base washed and silylated Celite 545.	Optimal flow as well as best separation of lipid classes. Same results as above, but more expensive. Same results as above, but requiring additional time for extraction and subsequent drying. Negligible removal of contaminants. Column flow rate severely limited. Poor separation of lipid classes during sequential elution. Poor separation of lipid classes during sequential elution. Poor separation of lipid classes during sequential elution. No separation of lipid classes. No improvement in class separation and slight retention of phospholipid.
C. To eliminate nonlipid coelution:	
Trap at base of column = 10 g CaHPO ₄ /Celite 545 1:9 ^b . Same trap as above, in 2:8 ratio. No trap.	Retains all but trace amounts of nonlipid. Column flow retarded; incomplete removal of lipid. Coelution of nonlipid >0.1% of tissue weight.

^aAll experiments done on 5-g tissue samples.

^bIndicates preferred procedure.

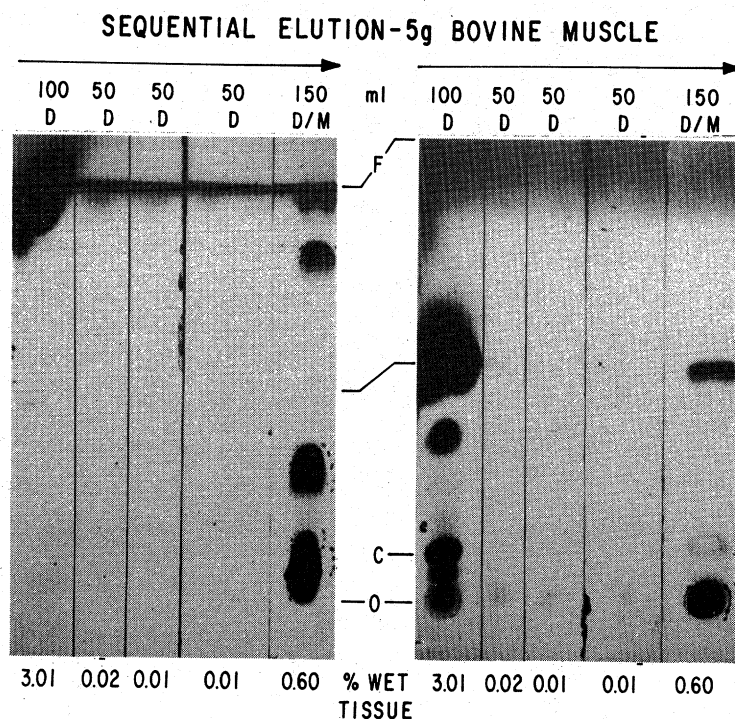


FIG. 1. TLC of 1% aliquots from sequential extracts of 5 g bovine muscle. TLC on Supelco L/S Redicoat plates, thickness 250 μ . Order of elution indicated with arrows. Eluants: dichloromethane (D) and then dichloromethane/methanol (D/M), 90:10. Left-hand plate developed in chloroform/methanol/acetic acid/water (85:15:10:3) to demonstrate absence of polar lipid ($R_f = 0-0.8$) until change of eluant. Visualization: same as Fig. 2. Neutral lipids appear near solvent front (F). Right-hand plate developed in hexane/ether/acetic acid (80:20:1) to demonstrate apparent exhaustion of neutral lipid (triglyceride $R_f = 0.5$; cholesterol indicated as "C") with initial eluant (D) and then emergence of additional neutral lipid upon change of eluant (D/M). Polar lipids remain at origin (O).

ances were read at 830 nm in a Spectronic 21-DV spectrophotometer (Bausch and Lomb; Fisher Scientific Company, King of Prussia, PA).

Determination of Calcium in Lipid Eluate

To determine whether calcium ion is leached from the column trap during solvent elution of lipid from the column, the eluates were analyzed for calcium by atomic absorption. Four parallel elutions were done: (a) standard procedure—5 g medium fat beef with column trap (trap = 9 g Celite 545 + 1 g $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$); (b) same beef, no trap; (c) no beef, but trap present; (d) no beef, no trap. Elution was performed sequentially, and only the second eluates—150 ml dichloromethane/methanol (90:10, v/v)—were collected for analysis. Each of the four eluates was brought to 200 ml in a volumetric flask, and aliquots were removed for phosphorus analysis. Solvent was evaporated from the remaining portions of eluate, and the residues then were hydrolyzed with 8 N nitric acid at 100 C for several hours. The

resulting solution was diluted to 1.6 N acid and analyzed for calcium content using a Perkin-Elmer Model 306 atomic absorption spectrophotometer.

RESULTS AND DISCUSSION

The conditions given for the dry column method (see Experimental) represent the optimal conditions that we have found for lipid isolation and were based on results from experiments designed to minimize neutral lipid carry-over into the polar lipid fraction and to prevent the coelution of nonlipid materials. The range of experiments applied to optimize those conditions is outlined in Tables I and II.

Although the neutral fraction from the sequential elution was free of polar lipid, a small but consistent amount of neutral lipid was isolated in the polar extracts using the best conditions found for the dry column method. Evidence for this neutral lipid carryover was shown by the following experiments. In the

TABLE III

Extractable Lipid^a by the Dry Column and CHCl₃/MeOH Methods

Tissue (5-g samples)	Dry column procedure				CHCl ₃ /MeOH extraction ^b
	Sequential elution			Isocratic elution	
	% Neutral lipid	Polar lipid ^c	Sum	Total lipid	Total lipid
Lean pork	L ^d : 4.97 (2) PL ^d : 0.00 (2)	0.82 (2) 0.64 (2)	5.79 0.64	5.94 ± 0.07 (4) 0.70 ± 0.00 (4)	5.77 ± 0.13 (4) 0.67 ± 0.02 (4)
Fatty beef	L: 29.35 (2) PL: 0.01 (2)	0.67 (2) 0.46 (2)	30.02 0.47	29.96 ± 0.04 (4) 0.49 ± 0.01 (4)	29.87 ± 0.22 (3) 0.50 ± 0.01 (3)
Medium beef	L: 10.08 (2) PL: — (0)	0.72 (2) 0.56 (2)	10.80 0.56	10.84 ± 0.11 (4) 0.62 ± 0.02 (4)	10.70 ± 0.11 (3) 0.61 ± 0.01 (3)
Lean beef	L: 3.43 ± 0.05 (5) PL: 0.01 (1)	0.80 ± 0.03 (5) 0.68 ± 0.01 (5)	4.23 0.69	4.36 ± 0.10 (7) 0.69 ± 0.03 (5)	4.38 ± 0.18 (6) 0.69 ± 0.01 (6)
4 Tissues, average	L: 11.96 PL: 0.01	0.75 0.58	12.71 0.59	12.78 0.62	12.68 0.62
Bovine subcutaneous adipose 1	L: PL:			82.12 ± 1.20 (5) 0.17 ± 0.00 (4)	81.56 ± 1.05 (4) 0.19 ± 0.01 (4)
Bovine subcutaneous adipose 2	L: PL:			85.26 ± 1.32 (4) 0.11 (1)	86.52 ± 1.84 (3) 0.11 (1)

^aAs percentage of total tissue weight.^b(6).^cIncludes ca. 10% carryover of neutral lipid.^dL = lipid; PL = phospholipid, calculated as 25 × % phosphorus. Standard deviations are included for runs in triplicate or greater; number of replicates is shown in parentheses.

sequential elution, the dichloromethane eluates (three 50-ml fractions) following the first 100 ml contained only traces of neutral lipid (0.5 mg or less). However, the change of solvent to the 90:10 mixture unexpectedly caused a release of neutral lipid into the eluate, and thus in the polar fraction, as seen by TLC analysis (Fig. 1).

This neutral lipid carryover consistently comprised ca. 10-15% of the polar fraction as determined by gravimetric analysis of polar fractions subsequently separated by TLC. The two neutral lipid mixtures, one from the dichloromethane eluate and the other separated from the polar fraction, also were compared by an examination of gas liquid chromatography (GLC) profiles of their fatty acid methyl ester derivatives. The GLC traces were similar in all major components and only minor differences could be observed. It would appear, therefore, that the neutral lipid carryover into the polar lipid fraction differs little in composition from the major neutral lipid fraction. The possibility that this holdback of neutral lipid (cf. Tables IB, IIB) may result from an affinity of Celite for neutral lipid was supported by the following experiment: tallow, containing no phospholipid, was mixed with dichloromethane and passed over a bed of 10 g of Celite 545 in

a 35-mm id chromatography column. The column was flushed with dichloromethane until no further lipid was detected in the eluate. Subsequent elution with 100 ml of the dichloromethane/methanol (90:10) mixture gave, after solvent removal, 3 mg of additional lipid. This amount of neutral lipid corresponds to that ordinarily found in the polar fraction from sequential extractions of muscle tissue.

To determine whether the calcium ion of the column trap (cf. Table IIC) was immobile, polar fraction eluates from sequential elutions of four columns were examined for calcium content by atomic absorption spectrophotometry. Eluates from blank columns—with or without the trap, but containing no bovine muscle—showed no calcium. Extract from a column with a trap had 1.38 μmol calcium/5 g muscle (and 33.4 μmol phosphorus/5 g muscle). Muscle extract from a column without a trap had 0.82 μmol calcium/6 g muscle. Therefore, 0.56 μmol calcium/5 g muscle, or 1 mol calcium for every 60 mol phosphorus (or phospholipid) was eluted from the trap. This amount of calcium is inconsequential for most lipid isolations, but is removable by ion exchange during a wash of the lipid extract with 0.88% aqueous KCl ("Folch wash" of reference 6).

The dry column extraction method, using

the preferred procedures (Experimental and Tables I and II), has been used on a variety of muscle and adipose tissues. Comparisons of the total lipid content and phospholipid recoveries obtained by the new method with those of a $\text{CHCl}_3/\text{MeOH}$ (modified Folch) procedure (6) are shown in Table III. The modified Folch procedure was used for comparison because it yielded a lipid extract that was free of nonlipid contaminants. Typical determinations were made in duplicate for sequential elutions and in quadruplicate for isocratic elutions by the dry column method. For purposes of comparison, replicate extractions by the $\text{CHCl}_3/\text{MeOH}$ method were done simultaneously with the dry column experiments. The results obtained by the two methods—isocratic elution by the dry column method vs the $\text{CHCl}_3/\text{MeOH}$ extrac-

tion—agree well for percentage lipid and percentage phosphorus in muscle tissue (Table III).

Results of the sequential elutions to obtain neutral and polar fractions also were compared with those of isocratic elutions by the dry column method (Table III). In all cases, the sum of the weights of the sequential fractions was nearly identical to the weight of the extract obtained by isocratic elution. All neutral lipid fractions were examined by TLC and phosphorus analysis for the presence of polar lipid, but only negligible amounts were detected. The phosphorus content of the polar fraction was in each case essentially identical to that found in the isocratic extract.

Analysis of excised adipose tissue, i.e., without skeletal muscle tissue, presented some difficulty by the sequential method. Class separations were in some cases erratic, and data for these fractions are not included in Table III. The separation of adipose tissue lipids into classes has long been recognized as a problem (8). Nevertheless, data shown in Table III confirm that the total lipid and the phospholipid contents of adipose tissue essentially were identical by the dry column (isocratic elution) and the $\text{CHCl}_3/\text{MeOH}$ methods.

To demonstrate the applicability of the dry column method to smaller sample sizes than those discussed in Table III, total lipid was extracted (isocratic elution) from 1-g tissue samples by $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (90:10). Although all lipid was removed in the first 20 ml of eluate, for the convenience of collecting in a volumetric flask, 25 ml would be suitable. Comparative results of parallel extractions of total lipid from bovine muscle were as follows: 5-g tissue samples, 4 runs: $3.96 \pm 0.02\%$ lipid and $0.67 \pm 0.02\%$ phospholipid; 1-g tissue samples, 4 runs: $4.15 \pm 0.23\%$ lipid and $0.65 \pm 0.03\%$ phospholipid.

Finally, a $\text{CHCl}_3/\text{MeOH}$ extract was separated by TLC (Fig. 2a) in parallel with three dry column extracts (Fig. 2b-d) of the same muscle. One aliquot (Fig. 2b) was from the isocratic elution with 150 ml of the 90:10 solvent mixture. Results of a sequential elution of a dry column are shown (Fig. 2c and d), in which d represents the neutral lipid fraction and c the polar fraction. Intensities of all separated components in Figure 2a were similar to those of Figure 2b. The large spots in the upper half of the plate are triglyceride. Cholesterol appears at $R_f = 0.4$. Seven subclasses of phospholipids are separated; phosphatidylcholine appears at $R_f = 0.1$ and phosphatidylethanolamine (ninhydrin positive) at $R_f = 0.2$. (Ninhydrin also detected only negligible nonlipid in both extracts.) Neutral lipid isolated

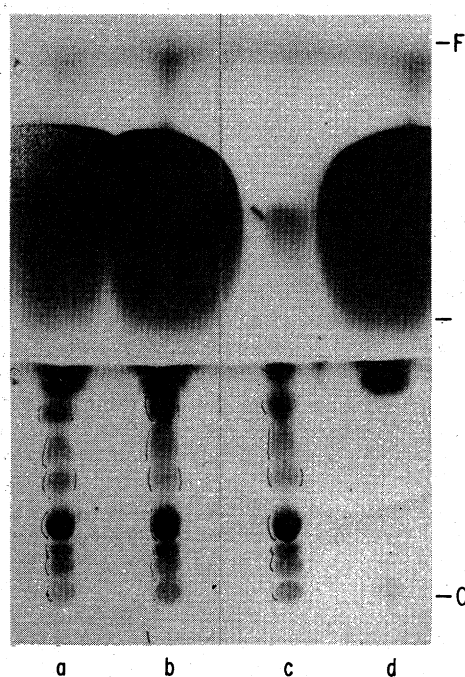


FIG. 2. TLC of 0.5% aliquots of modified Folch extract (a) and dry column extracts (b-d) of 5 g bovine muscle. Column b = total lipid eluate from isocratic elution of dry column; d and c = neutral fraction and polar fraction, respectively, from sequential elution of dry column. TLC on Analtech (Newark, DE) Silica Gel G Uniplates, thickness 250 μ . Double development, initially with chloroform to indicated solvent front F in order to draw the heavy triglyceride spot away from the remaining lipid, and then with chloroform/methanol/water (65:35:5) about half way up to separate the remaining lipids. Visualization: ninhydrin (positive spots indicated with right-hand parentheses), then Phospray (... left-hand parentheses), then charring. Cholesterol appears at $R_f = 0.4$; "O" = origin.

by sequential elution is free of polar lipid. The polar lipids, Figure 2c, are identical in composition to the polar lipids seen in columns a and b, along with a trace of triglyceride ($R_f = 0.7$) and cholesterol ($R_f = 0.4$).

In summary, though quantification of lipid components and determination of their structural integrity await further study, the TLC patterns, the weight recovery, and the phosphorus analysis data indicate that the dry column method may be an acceptable and advantageous alternative to the traditional chloroform/methanol extraction methods for lipid analysis of muscle and adipose tissue.

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